II. REMARKS

Formal Matters

Claims 1-21 are pending after entry of the amendments set forth herein.

Claims 8-12 were examined and were rejected. Claims 1-7 and 13-20 were withdrawn from consideration.

New claim 21 is added. Support for new claim 21 is found in the claims as originally filed, and throughout the specification, in particular at the following exemplary locations: page 21, paragraph 0077. Accordingly, no new matter is added.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

PTO 1449/SB-08A form

Applicants respectfully request that the Examiner initial and return the PTO 1449 and PTO SB/08A forms submitted with the Information Disclosure Statements filed on June 28, 2001 and June 28, 2002, respectively, in this application, thereby indicating that the references cited therein have been reviewed and made of record.

Rejection under 35 U.S.C.§112, second paragraph

Claims 8-12 were rejected under 35 U.S.C.§112, second paragraph, as allegedly indefinite.

Claim 12

The Office Action stated that the term "cell-based method" is unclear. Applicants respectfully traverse the rejection.

The term "cell-based method" is clear, and refers to a method conducted using a living cell. The term "cell-based method" (and similar terms such as "cell-based assay" and "cell-based system") are understood in the art to refer to methods conducted using a living cell. Indeed, numerous, presumably valid, U.S. patents have issued with claims that recite such terms. See, e.g., U.S. Patent Nos. 6,432,654; 6,365,365; 6,262,340; 6,221,578; 6,200,769; 6,060,263; 5,981,207, 5,942,387; and 5,780,258. Thus, this term does not render claim 12 indefinite. Therefore, claim 12 need not be amended.

Claims 8-12

The Office Action stated that claims 8-12 are indefinite in reciting that a change in activity of DNA-PK "indicates" that the agent modulates a biological activity of DNA-PK. The Office Action stated that the term "indicates" is not as definitive as the term "identify" as recited in the preamble.

Applicants respectfully traverse the rejection. The test for whether a claim complies with the requirements of 35 U.S.C.§112, second paragraph is not whether a term is "not as definitive" as another term in the claim. Under 35 U.S.C.§112, second paragraph, the claims must set forth the subject matter that applicants regard as their invention; and the claims must particularly point out and distinctly define the metes and bounds of the subject matter that will be protected by the patent grant. Claims 8-12 meet these requirements. Indeed, a numerous, presumably valid, U.S. patents have issued with claims that recite methods for identifying compounds with a certain activity, which claims include a phrase similar to "wherein an increase or a decrease in the biological activity of DNA-PK indicates that the agent modulates a biological activity of DNA-PK." See, e.g., U.S. Patent Nos. 5,976,808; 6,096,499; 6,287,782; 6,352,830; and 6,417,208. Thus, claims 8-12 meet the requirements of 35 U.S.C.§112, second paragraph, and need not be amended.

Conclusion as to the rejections under 35 U.S.C.§112, second paragraph

Applicants submit that the rejection of claims 8-12 under 35 U.S.C. §112, second paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Rejection under 35 U.S.C.§103

Claims 8-11 were rejected under 35 U.S.C.§103 as allegedly unpatentable over Muller et al. ((1998) *Blood* 92:2213-2219; "Muller") in view of Finnie et al. ((1995) *Proc. Natl. Acad. Sci. USA* 92:320-324; "Finnie"), further in view of Lees-Miller et al. (1990) *Mol. Cell. Biol.* 10:6472-6481; "Lees-Miller"), and further in view of Han et al. ((1996) *J. Biol. Chem.* 271:14098-14104; "Han").

Claim 12 was rejected under 35 U.S.C.§103 as allegedly unpatentable over Klinman et al. ((1996) *Proc. Natl. Acad. Sci. USA* 93:2879-2883; "Klinman") in view of Munoz et al. ((1998) *Mol. Cell. Biol.* 18:5797-5808; "Klinman"), further in view of Lees-Miller, and further in view of Krieg et al. ((1995) *Nature* 374:546-549; "Krieg").

Claims 8-11 over Muller in view of Finnie, Lees-Miller, and Han

The Office Action stated:

1) Muller teaches a method (as described by Finnie) for identifying an agent that modulates a biological activity of DNA-PK;

2) Muller does not specifically teach that the nucleic acid molecule to which DNA-PK binds is an immunostimulatory nucleic acid; and

3) Lees-Miller teaches that double-stranded nucleic acid molecules with a variety of sequences and structures stimulate the activity of DNA-PK, and of these, the most effective is an oligonucleotide that contains multiple GC repeats.

The Office Action concluded that it would have been obvious to use the poly-GC oligonucleotide taught by Lees-Miller in the assay method of Muller in order to maximize the intensity of the DNA-PK signal to provide an assay method with the highest possible specificity and sensitivity. Applicants respectfully traverse the rejection.

Comments regarding the instant invention

The instant invention relates to the unexpected observations that nucleic acid molecules previously defined as immunomodulatory nucleic acid molecules bind to Ku antigen and activate DNA-PKcs; immunomodulatory nucleic acid molecules activate the anti-apoptotic PI3P-dependent kinase Akt; and immunomodulatory nucleic acid molecules induce an anti-apoptotic response in eukaryotic cells. These observations were published after the priority date of the instant application by Applicants in Chu et al. ((2000) *Cell* 103:909-918, a copy of which is provided herewith as Exhibit 1). The observation that immunomodulatory nucleic acid molecules bind to Ku antigen and activate DNA-PKcs lead to the development of a method for identifying an agent that modulates a biological activity of DNA-PK, the method comprising adding an agent to be tested to a sample, the sample comprising DNA-PK and an immunomodulatory nucleic acid molecule, and detecting a biological activity of the DNA-PK. None of the cited art discloses or suggests that immunomodulatory nucleic acids bind to and activate DNA-PK.

Comments regarding the cited art

The cited art does not disclose or suggest a method of identifying an agent that modulates a biological activity of DNA-PK, the method comprising adding an agent to be tested to a sample, the sample comprising DNA-PK and an immunomodulatory nucleic acid molecule, and detecting a

biological activity of the DNA-PK.

None of the cited art discloses or suggests a method involving a sample that comprises DNA-PK and an immunomodulatory nucleic acid molecule. As the Office Action acknowledged, Muller does not teach that the nucleic acid molecule to which the DNA-PK binds is an immunostimulatory nucleic acid. Lees-Miller also does not disclose or suggest a method involving a sample that comprises DNA-PK and an immunomodulatory nucleic acid molecule.

The Office Action stated that Lees-Miller teaches that a double-stranded oligonucleotide containing multiple GC repeats was the most effective in stimulating DNA-PK activity. The Office Action stated that an oligonucleotide comprising multiple GC repeats was known to those of skill in the art at the time the application was filed to have immunostimulatory effects. However, the cited art does not support the assertion that an oligonucleotide comprising multiple GC is an immunostimulatory nucleic acid. If the Examiner knows of references that would support such an assertion, Applicants respectfully request that the Examiner provide such.[1]

The Office Action further stated that an oligonucleotide comprising multiple GC repeats satisfies the definition of immunostimulatory nucleic acid presented in the specification on page 1, paragraph 4. However, the specification on page 1, paragraph 4, states that oligodeoxynucleotides containing CpG dinucleotides in a particular base context are immunostimulatory, and discusses the art relating to immunostimulatory nucleic acids. The specification further discusses the sequences of immunostimulatory nucleic acids. Specification, page 20, paragraph 75, to page 23, paragraph 82. The cited art does not disclose that oligonucleotides comprising GC repeats are immunostimulatory.

The cited art does not teach or suggest a method for identifying an agent that modulates a biological activity of DNA-PK, comprising adding an agent to be tested to a sample, the sample comprising DNA-PK and an immunomodulatory nucleic acid molecule, and detecting a biological activity of DNA-PK. The cited art does not teach or suggest that DNA-PK binds to and is activated by an immunomodulatory nucleic acid. Accordingly, the cited art cannot render the instant invention as recited in claim 8 obvious.

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[1] Under 37 C.F.R. §1.104(d)(2), when a rejection is based on facts within the personal knowledge of an employee of the Office, the data shall be as specific as possible, and the reference must be supported, when called for by the applicant, by the affidavit of such employee.

The Office Action further stated that Muller teaches the embodiment, recited in claim 9, in which the biological activity of DNA-PK is binding to an immunomodulatory nucleic acid molecule, where the sequence of oligonucleotides is taught in Han. However, as discussed above, claim 8 is non-obvious over Muller in view of Lees-Miller, as neither Muller nor Lees-Miller discloses contacting a test agent with a sample comprising a immunomodulatory nucleic acid molecules and DNA-PK. A discussion of DNA-PK binding to oligonucleotides does not cure the deficiency of Muller, and therefore does not suffice to render claim 9, which depends from claim 8, obvious.

The Office Action further stated that Muller teaches the embodiment, recited in claim 10, in which the method is a cell-free method and the immunomodulatory nucleic acid molecule is detectably labeled. However, as discussed above, claim 8 is non-obvious over Muller in view of Lees-Miller, as neither Muller nor Lees-Miller discloses contacting a test agent with a sample comprising a immunomodulatory nucleic acid molecules and DNA-PK. Muller does not disclose or suggest use of an immunomodulatory nucleic acid. A discussion of a cell-free method, or a detectably labeled oligonucleotide does not cure the deficiency of Muller, and therefore does not suffice to render claim 10, which depends indirectly from claim 8, obvious.

The Office Action further stated that Muller teaches the embodiment, recited in claim 11, in which the biological activity of DNA-PK is activation of DNA-PK kinase activity. However, as discussed above, claim 8 is non-obvious over Muller in view of Lees-Miller, as neither Muller nor Lees-Miller discloses contacting a test agent with a sample comprising a immunomodulatory nucleic acid molecules and DNA-PK. Merely disclosing activation of DNA-PK kinase activity does not suffice to render claim 11, which depends from claim 8, obvious.

Claim 12 over Klinman in view of Munoz, Lees-Miller, and Krieg

The Office Action stated that Klinman teaches a method for identifying an agent that modulates the production of IL-6 and IL-12, in which immunomodulatory nucleic acid molecules were combined with $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, and NK cells to form a test sample. The Office Action stated that these cell types were known to those of skill in the art to contain DNA-PK, citing Munoz. The Office Action further stated that agents were added to the test sample and a biological activity (production of IL-6, IL-12, IFN-γ, IgM) was detected. The Office Action stated that Klinman does not specifically teach that the method for treating cells with oligonucleotides containing CpG

motifs and measuring production of IL-6 and IL-12 was an assay of DNA-PK activity. The Office Action stated that it was known that oligonucleotides containing CpG motifs were potent activators of DNA-PK, citing Lees-Miller. The Office Action stated that because treatment of cells with oligonucleotides containing CpG motifs resulted in both an activation of DNA-PK and secretion of IL-6 and IL-12, it would have been obvious to one of ordinary skill in the art to use IL-6 and IL-12 production as an indirect measure of DNA-PK activation by oligonucleotides containing CpG motifs. Applicants respectfully traverse the rejection.

As discussed above, Applicants made the unexpected observation that nucleic acid molecules previously defined as immunomodulatory nucleic acid molecules bind to Ku antigen and activate DNA-PKcs. None of the cited art discloses or suggests that immunomodulatory nucleic acids bind to and activate DNA-PK.

The Office Action stated that Klinman teaches an assay method for identifying an agent that modulates production of IL-6 and IL-12. The Office Action stated that agents such as antibodies against IL-6, IL-12, IFN-γ, GMCSF, and control antibodies were added to a test sample containing cells and immunomodulatory nucleic acid molecules. However, an assay for identifying an agent that modulates production of IL-6 and IL-12 does not suggest a method for identifying an agent that modulates DNA-PK activity. The agents cited in the Office Action, i.e., IL-6, IL-12, IFN-γ, GMCSF, and control antibodies, are not known to have any effect on DNA-PK activity. The instant invention as claimed does not relate to a method of identifying an agent that modulates production of IL-6 and IL-12. Instead, IL-6 and IL-12 production are a readout for effects of an agent on DNA-PK activity.

Lees-Miller does not cure the deficiency of Klinman. As discussed above, Lees-Miller does not disclose or suggest that an immunomodulatory nucleic acid binds to and activates DNA-PK activity. Until the present application was filed, the art did not disclose or suggest that an immunomodulatory nucleic acid binds to and activates DNA-PK activity. Thus, Klinman, alone or in combination with the other cited art, cannot render the instant invention as claimed obvious.

Conclusion as to the rejections under 35 U.S.C.§103

Applicants submit that the rejections of claims 8-12 under 35 U.S.C. §103 have been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejections.

III. CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number UCAL168.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Jan. 30, 2003

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DNA-PKcs Is Required for Activation of Innate Immunity by Immunostimulatory DNA

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Summary

Bacterial DNA and related synthetic immunostimulatory oligodeoxyribonucleotides (ISS-ODN) stimulate innate immunity. However, the molecular recognition mechanism that initiates signaling in response to bacterial DNA and ISS-ODN has not been identified. Herein, we demonstrate that administration of bacterial DNA and ISS-ODN to mice lacking the catalytic subunit of DNA-PK (DNA-PKcs) and in vitro stimulation of BMDM from these mice result in defective induction of IL-6 and IL-12. Further analysis using BMDM of IKKβ$^{-/-}$ revealed that both DNA-PKcs and IKKβ are essential for normal cytokine production in response to ISS-ODN or bacterial DNA. ISS-ODN and bacterial DNA activate DNA-PK, which in turn contributes to activation of IKK and NF-κB. These results reveal a novel role of DNA-PKcs in innate immune responses and a link between DNA repair and innate immunity.

Introduction

In addition to the well-established role of bacterial cell wall components in activation of innate immunity (reviewed by Aderem and Ulevitch, 2000), it has been recognized that bacterial DNA also has profound immunostimulatory effects (Klinman et al., 1999; Van Uden and Raz, 1999; Wagner, 1999). Tokunaga and colleagues had initially demonstrated that particular sequences of oligodeoxyribonucleotides (ODN) derived from the mycobacteria genome elicit anti-tumor activity (Tokunaga et al., 1984). Subsequent analysis of shorter overlapping ODNs and insertion of active sequences into otherwise inactive ODNs suggested that palindromic hexamers, each of which contains nonmethylated CpG dinucleotides, are responsible for this immune activation (Yamamoto et al., 1992). Since this initial characterization, further studies have determined the optimal sequences for immunostimulatory activity leading Krieg and coworkers to define unmethylated 5′-Pur-Pur-CpG-Pyr-Pyr-3′ motif as the minimal active sequence that triggers IL-6 production (Krieg et al., 1995).

More recent studies have demonstrated that bacterial DNA and related ISS-ODNs activate essential cellular components of innate immunity. Upon such stimulation, macrophages, monocytes, and dendritic cells secrete proinflammatory cytokines such as IL-6 and IL-12 (Klinman et al., 1996), and overexpress costimulatory molecules such as B7 and CD40 (Bauer et al., 1999; Martin-Orozco et al., 1999). These immunomodulatory properties of ISS-ODN were proposed to mediate induction of Th1 responses to both DNA (Sato et al., 1996) and protein-based vaccines (Roman et al., 1997; reviewed by Van Uden and Raz, 1999), as well as to induce cross-presentation of exogenous, soluble antigens, and to prime cytotoxic T cell responses (Cho et al., 2000).

Curiously, methylation at the C-5 position of the cytosine in the CpG dinucleotide ($^{5\text{-methyl}}$CpG) reduces this immunostimulatory activity (Krieg et al., 1995). This phenomenon is particularly interesting because genomic DNA of vertebrates contains highly methylated cytosines (70%) of the CpG dinucleotide, whose frequency is already reduced about 5-fold from the expected frequency (CpG suppression). By contrast, bacterial genomic DNA contains the expected frequency of CpG dinucleotide, which is usually not methylated on the cytosine (Bird, 1993). These differences between bacterial/mycobacterial and mammalian DNA led to the hypothesis that the mammalian innate immune system has evolved to respond to a bacterial-specific feature of DNA structure (i.e., pattern recognition system) through unique and yet unknown pattern recognition receptors (Medzhitov and Janeway, 1997). It was speculated that a putative ISS-ODN receptor would detect the DNA of invading microbial pathogens and elicit the "immunological danger/alarm signal" necessary for protection of the mammalian host (Roman et al., 1997; Klinman et al., 1999; Wagner, 1999).

The signaling pathways that mediate the immunostimulatory properties of ISS-ODN have also been investigated. It was reported that bacterial DNA and ISS-ODNs do not induce tyrosine phosphorylation or an increase in either inositol triphosphates or intracellular Ca^{2+} in responding cells (Krieg et al., 1995). In contrast, the NF-κB and MAP kinase signaling pathways were shown to be activated (Stacey et al., 1996; Hacker et al., 1998). Inhibition of NF-κB activation prevents IL-6 production in response to ISS-ODN (Yi et al., 1998). However, the molecular link that leads from fragmented bacterial DNA or ISS-ODN to NF-κB activation remains to be elucidated.

As ISS-ODNs are taken up into cells, they are thought to signal via their interaction with certain intracellular molecules (Hacker et al., 1998), which we postulated

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might involve intracellular protein kinases known to be activated by DNA. One such protein kinase is the DNA-dependent protein kinase (DNA-PK). DNA-PK is a member of phosphatidlinositol 3 (PI3) kinase-like family that also includes ATM, FRAP, and FRP1 (Hartley et al., 1995; Smith and Jackson, 1999). DNA-PK can be detected in both the nucleus and cytoplasm (Carter et al., 1990; Danska et al., 1996; Koiker et al., 1999; Nilsson et al., 1999). In the nucleus, DNA-PK plays a pivotal role in repair of DNA double-stranded breaks created by environmental insults, such as ionizing radiation (Hartley et al., 1995; Kirchgessner et al., 1995; Smith and Jackson, 1999) or by intrinsic cellular processes such as programmed DNA rearrangements during lymphocyte differentiation (e.g., VDJ recombination) (Smith and Jackson, 1999). In contrast, the cytoplasmic functions of DNA-PK are unclear. At physiological salt concentrations, DNA-PK requires two components for activity: a DNA binding protein, Ku, and a catalytic subunit, DNA-PKcs (Gottlieb and Jackson, 1993). Ku is a heterodimer of 70 and 86 kDa polypeptides that binds to double-stranded DNA ends, nicks, single-stranded DNA, or transitions between double- and single-strand DNA (Mimori and Hardin, 1986; Falzon et al., 1993). DNA-PKcs has double- and single-strand DNA binding domains and is activated by both double- and single-stranded DNA ends (Leuther et al., 1999; Hammarsten et al., 2000).

We hypothesized a role for DNA-PK in the induction of innate immunity by bacterial DNA and ISS-ODNs, and obtained biochemical and genetic evidence that DNA-PKcs is indeed required for signaling events that lead to induction of innate cytokines, such as IL-6 and IL-12. We demonstrate that bacterial DNA and ISS-ODN activate DNA-PKcs, which can phosphorylate the IKKβ subunit of the IKK complex to cause NF-κB activation. Both DNA-PKcs and IKKβ are essential for induction of cytokines in response to bacterial DNA and ISS-ODN.

Results

The experiments described in this study were performed with single-stranded (ss), 22 nucleotide long, phosphothioate (ps) ODNs. These are termed as ISS-ODN where ISS stands for immunostimulatory sequence, M-ODN where M stands for mutated, and cont-ODN where cont stands for control. Single-stranded phosphodiester (po) ODNs are termed po-ISS-ODN or po-M-ODN whereas double-stranded (ds) po-ODNs are termed po-ds-ISS-ODN or po-ds-M-ODN (for more details, see Experimental Procedures).

DNA-PKcs Is Required for Innate Cytokine Induction by Bacterial DNA and ISS-ODN

Bone marrow–derived macrophages (BMDM) respond to ISS-ODN by secreting high levels of IL-6 and IL-12 (Martin-Orozco et al., 1999). Initially, BMDM from DNA-PKcs-deficient mice (Kurimasa et al., 1999) were used to explore the possible role of DNA-PKcs in induction of these innate cytokines by ISS-ODN. Very low levels of IL-6 and IL-12 were produced by DNA-PKcs-deficient BMDM upon ISS-ODN stimulation in comparison to wild-type (wt) BMDM (Figures 1A and 1B). In contrast, DNA-PKcs$^{-/-}$ BMDM exhibited normal induction of IL-6 and IL-12 in response to LPS stimulation (Figures 1A and 1B).

Since phosphothioate (ps) ODNs differ structurally from phosphodiester (po) ODNs, we compared the response of ISS-ODN to po-ISS-ODN, po-ds-ISS-ODN (unmethylated or methylated), LPS free bacterial DNA (*E. coli*), methylated *E. coli* bacterial DNA, or to LPS free calf thymus DNA. Similar activity profile was observed for po-ds-ISS-ODN and bacterial DNA in wt BMDM (Figures 1C and 1D) while po-ISS-ODN was less effective (Figures 1C and 1D). As expected, calf thymus DNA and methylated bacterial DNA induced a several-fold less IL-6 and IL-12 as compared to unmethylated bacterial DNA (data not shown). BMDM from DNA-PKcs-deficient mice were also defective in induction of IL-6 and IL-12 in response to po-ISS-ODN, po-ds-ISS-ODN, and bacterial DNA (Figures 1C and 1D), indicating that DNA-PKcs is required for induction of IL-6 and IL-12 by synthetic (ps) and natural forms (po) of ISS-enriched DNAs (i.e., bacterial DNA).

We then determined whether the lack of ISS-ODN responsiveness in DNA-PKcs-deficient BMDM was due to a defect in mRNA induction. Little induction of IL-6 and IL-12 mRNAs in response to ISS-ODN was observed in DNA-PKcs-deficient BMDM (Figure 1E). In contrast, DNA-PKcs-deficient BMDM exhibited normal cytokine mRNA induction in response to LPS stimulation.

To determine the requirement for DNA-PKcs in the induction of IL-6 and IL-12 by ISS-ODN, po-ISS-ODN, ps-ds-ISS-ODN, bacterial and calf thymus DNAs in vivo, we injected these DNAs into wt and DNA-PKcs$^{-/-}$ mice. The levels of IL-6 and IL-12 mRNAs in liver or spleen were examined by RT-PCR. IL-6 or IL-12 mRNA levels were detected in the liver or the spleen of wt controls, but were lacking in the same organs in DNA-PKcs-deficient mice (Figure 1F). Only minute amounts of mRNAs were observed in response to calf thymus DNA injection into wt mice (Figure 1F).

DNA-PKcs is a member of PI3K family and its enzymatic activity is blocked by PI3K inhibitors such as wortmannin (Wm) at high concentrations, or Ly294002 (Ly) (Hartley et al., 1995; Smith and Jackson, 1999). To further establish the role of DNA-PKcs in the induction of IL-6 and IL-12 by ISS-ODN, we examined the effects of Wm and Ly on these responses. High concentrations of Wm (>100 nM) significantly inhibited the induction of IL-6 and IL-12 by ISS-ODN (Hartley et al., 1995) (Figures 2A and 2B). Ly also blocked IL-6 and IL-12 induction by ISS-ODN (data not shown). In contrast, both Wm and Ly did not inhibit LPS-induced secretion of IL-6 and IL-12 (Figures 2A and 2B and data not shown).

The ATM gene product, which is also a member of the PI3K family, is functionally related to DNA-PKcs and its kinase activity is also Wm and Ly sensitive (Hartley et al., 1995; Xu et al., 1996). We therefore examined the induction of IL-6 and IL-12 in ATM-deficient mice. As shown in Figures 2C and 2D, normal induction of IL-6 and IL-12 by ISS-ODN was observed in ATM-deficient BMDM, excluding a role for ATM in ISS-induced activation of innate immunity.

IKKβ Is Required for BD and ISS-ODN-Induced Activation of NF-κB

Previous study had demonstrated activation of NF-κB by ISS-ODN (Stacey et al., 1996). However, the mechanism that drives this response is largely unknown. To

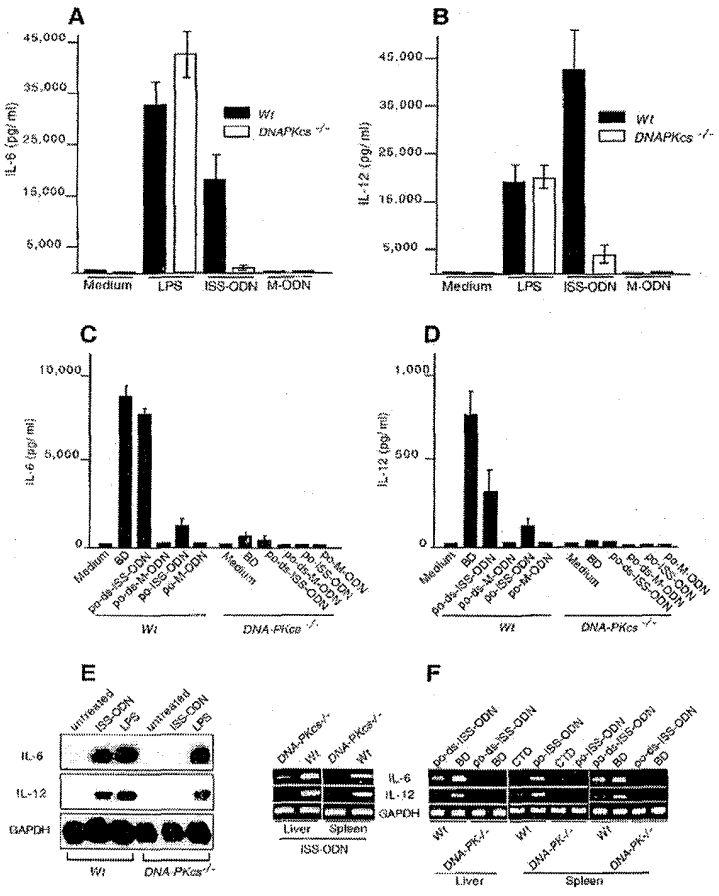

Figure 1. DNA-PKcs Is Required for Induction of IL-6 and IL-12 by ISS-ODN and Bacterial DNA

(A and B) Bone marrow–derived macrophages (BMDM) from wt or *DNA-PK*$^{-/-}$ mice were treated with ISS-ODN (5 μg/ml), M-ODN (5 μg/ml), LPS (1 μg/ml) or left untreated for 24 hr. IL-6 and IL-12 levels were determined in the supernatants by ELISA.

(C and D) BMDM from wt or *DNA-PK*$^{-/-}$ mice were treated with po-ds-ISS-ODN, po-ds-M-ODN, po-ISS-ODN, or po-M-ODN (10 μg/ml each), bacterial DNA (BD, 15 μg/ml) or calf thymus DNA (CTD, 15 μg/ml), or left untreated for 24 hr. IL-6 and IL-12 levels were determined by ELISA.

(E) Northern blot analysis. Total RNA was isolated from wt or *DNA-PKcs*$^{-/-}$ BMDM untreated or treated with ISS-ODN (5 μg/ml) or LPS (1 μg/ml) after 6.5 hr and examined for expression of IL-6, IL-12, and GAPDH mRNAs.

(F) In vivo analysis of gene expression. ISS-ODN (20 μg in PBS/mouse), po-ISS-ODN (100 μg), or po-ds-ISS-ODN (100 μg), bacterial DNA (BD, 50 μg) or calf thymus DNA (CTD, 50 μg) were i.v. injected into wt or *DNA-PKcs*$^{-/-}$ mice. After 3 hr total, RNA was extracted from the spleen or liver and subjected to RT-PCR analysis.

resolve this issue, we initially evaluated whether ISS-ODN activated IKK, which is essential for NF-κB activation by proinflammatory stimuli (reviewed by Karin and Delhase, 2000). We observed maximal IKK activation 30 min post-ISS-ODN incubation, which lasted for about 4 hr in wt BMDM (Figure 3A). While bacterial DNA and po-ISS-ODN induced IKK activation similar to ISS-ODN, little increase in IKK activity was observed with M-ODN or calf thymus DNA (Figure 3A). Optimal IKK activation was observed at an ISS-ODN concentration of 0.65 μg/ml, with little IKK activation in response M-ODN (Figure 3B).

We used BMDM isolated from *IKKβ*$^{-/-}$ *Tnfr1*$^{-/-}$ mice (Z.-W. L and M. K., unpublished data) to determine the requirement of IKK activity, which is highly reduced in these animals. The absence of IKKβ prevented IKK and NF-κB activation by ISS-ODN as well as LPS (Figure 3C) and significantly reduced the induction of IL-6 and IL-12 (Figures 3D and 3E).

DNA-PKcs Acts Upstream to IKK

The data presented above indicate that both DNA-PKcs and IKK are required for ISS-induced IL-6 and IL-12. We therefore examined the dependence of IKK activation by ISS-ODN on DNA-PKcs. While incubation of wt BMDM with ISS-ODN resulted in robust IKK activation, little increase in IKK activity was observed in similarly treated DNA-PKcs$^{-/-}$ BMDM (Figure 4A). As a result, DNA-PKcs$^{-/-}$ BMDM also exhibited impaired NF-κB activation upon treatment with ISS-ODN (Figure 4A). By contrast, DNA-PKcs-deficient BMDM were fully responsive to LPS or TNFα. Furthermore, we examined the dependence of IKK activation by bacterial DNA and po-ISS-ODN on DNA-PKcs. As expected, activation of IKK by bacterial DNA or po-ISS-ODN was largely reduced in DNA-PKcs$^{-/-}$ BMDM as compared to their wt controls (Figure 4B).

To determine whether DNA-PKcs activity is required for IKK activation, we used the PI3K inhibitor Wm. As found for IL-6 and IL-12 production (Figures 2C and 2D), only high concentrations of Wm (250 nM and above) significantly inhibited IKK activation in wt BMDM by ISS-ODN (Figure 4C). Even at 1000 nM, Wm had no effect on IKK activation by TNFα, while at lower concentrations (50–100 nM), Wm significantly inhibited IKK activation by PGDF (data not shown). We also compared ISS-ODN-induced of IKK and NF-κB activation in BMDM from wt or ATM-deficient mice (Xu et al., 1996). As shown in Figure 4D, no differences were observed for ISS-ODN-induced IKK or NF-κB activation between wt and ATM-deficient BMDM, excluding a role of ATM in this signaling.

Taken together, these results indicate that DNA-PKcs

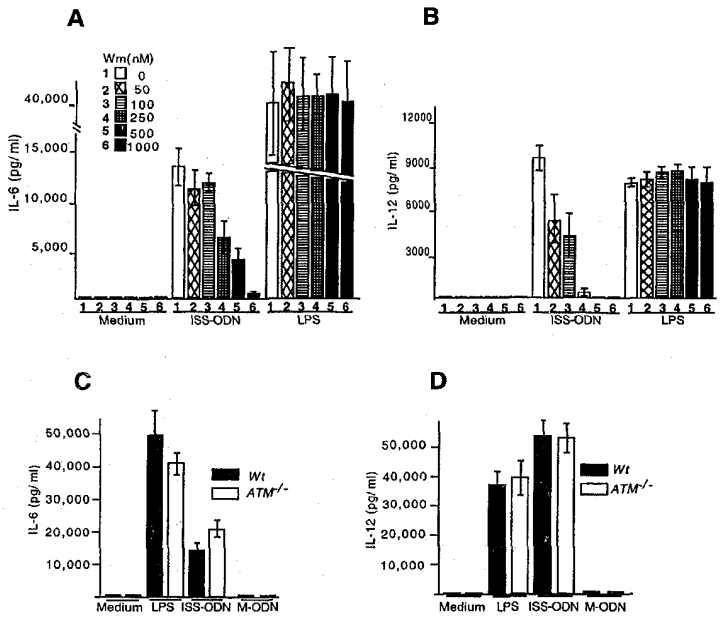

Figure 2. DNA-PK but Not ATM Activity Is Necessary for IL-6 and IL-12 by ISS-ODN

(A and B) BMDM from wt mice were treated with ISS-ODN (5 μg/ml) or LPS (1 μg/ml) in the presence of various concentrations of wortmannin (Wm). The levels of IL-6 and IL-12 in the supernatants were determined by ELISA.

(C and D) BMDM from either wt or *ATM*$^{-/-}$ mice were treated with ISS-ODN (5 μg/ml), M-ODN (5 μg/ml), LPS (1 μg/ml), or left untreated for 24 hr. IL-6 and IL-12 levels in the supernatants were determined by ELISA.

acts upstream to IKK and is specifically required for IKK activation by synthetic (ps) and natural forms (po) of ISS-enriched DNAs.

ISS-ODN Directly Activates DNA-PK

We investigated whether ISS-ODN can directly activate DNA-PK in vitro. The ability of an ISS-ODN containing the active CpG motif (5′-pur-pur-CpG-pyr-pyr-3′, i.e., 5′-AACGTT-3′) to specifically stimulate phosphorylation of the N-terminal portion of p53 (Wang and Eckhart, 1992) was compared to a battery of mutated ODNs, which include: (1) a methylated C in the CpG dinucleotide, (2) a CpC base pair instead of the CpG dinucleotide core, (3) a GpG base pair instead of the CpG dinucleotide core, (4) an ApT basepair instead of the CpG dinucleotide core, and (5) a TTCC instead of the AACG sequence of the CpG motif (see above). None of the mutant ODNs induced significant secretion of IL-6 or IL-12 upon stimulation of BMDM in vitro (data not shown). Only the ISS-ODN stimulated DNA-PK activity (Figure 5A) whereas none of the mutant ODNs, which were devoid of biological activity, led to substantial increase in DNA-PK activity.

Next, we investigated the ability of po-ISS-ODN to activate DNA-PK in vitro. Unlike ISS-ODN (Figure 4A), po-ISS-ODNs weakly activated DNA-PK only at higher concentrations (50–100 ng/reaction) (Figure 5B). By contrast, po-ds-ISS-ODN was almost as potent as ISS-ODN in activating this enzyme (Figure 5C). Methylated ISS-ODN (AA*CGTT) and methylated po-ISS-ODN (AA*CGTT) were weaker DNA-PK activators than their unmethylated counterparts (Figures 5A and 5B, respectively).

In addition, we evaluated the ability of bacterial DNA, methylated bacterial DNA, and calf thymus DNA to activate DNA-PK. To use the same equimolar amount of the various DNA preparations in the DNA-PK assays, we first measured the 5′ free ends using T4 DNA polynucleotide kinase in the DNA preparations. For bacterial DNA, this labeling yielded 6.29×10^5 cpm/0.1 μg and 4.1×10^6 cpm/1 μg of DNA, and for calf thymus DNA it yielded 8.58×10^5 cpm/0.1 μg and 4.2×10^6 cpm/1 μg of DNA. Under these conditions, calf thymus DNA was a weaker activator of DNA-PK than bacterial DNA (Figure 5D), while methylated bacterial DNA was a less potent DNA-PK activator than unmethylated bacterial DNA (Figure 5E).

To further determine whether ISS-ODN, po-ISS-ODN, or bacterial DNA activate DNA-PK in cells, we treated BMDM from either wt or DNA-PKcs-deficient mice with ISS-ODN, po-ISS-ODN, bacterial DNA, or LPS as a control. Considerable DNA-PK activity, as measured by immune-complex kinase assay, was found after a 30 min incubation with ISS-ODN, po-ISS-ODN, or bacterial DNA, which peaked after 1 hr (Figure 5F). Little or no DNA-PK activity was detected in DNA-PKcs-deficient BMDM, and LPS had no effect on DNA-PK activity even in wt cells.

DNA-PK Phosphorylates IKKβ

To explore a role of ISS-activated DNA-PK in IKK activation we tested whether affinity-purified DNA-PK can directly activate recombinant IKKα or IKKβ purified from Sf9 cells (Zandi et al., 1998). Recombinant IKKα and IKKβ display considerable basal kinase activity (Zandi et al., 1998; Chu et al., 1999), but incubation of IKKβ with DNA-PK in the presence of ISS-ODN further increased its kinase activity measured by IκB phosphorylation (Figure 6A). Furthermore, although DNA-PKcs phosphorylated IκBα, that activity was considerably lower than that achieved by IKKβ plus DNA-PK. Only a small enhancement of IκB kinase activity was found

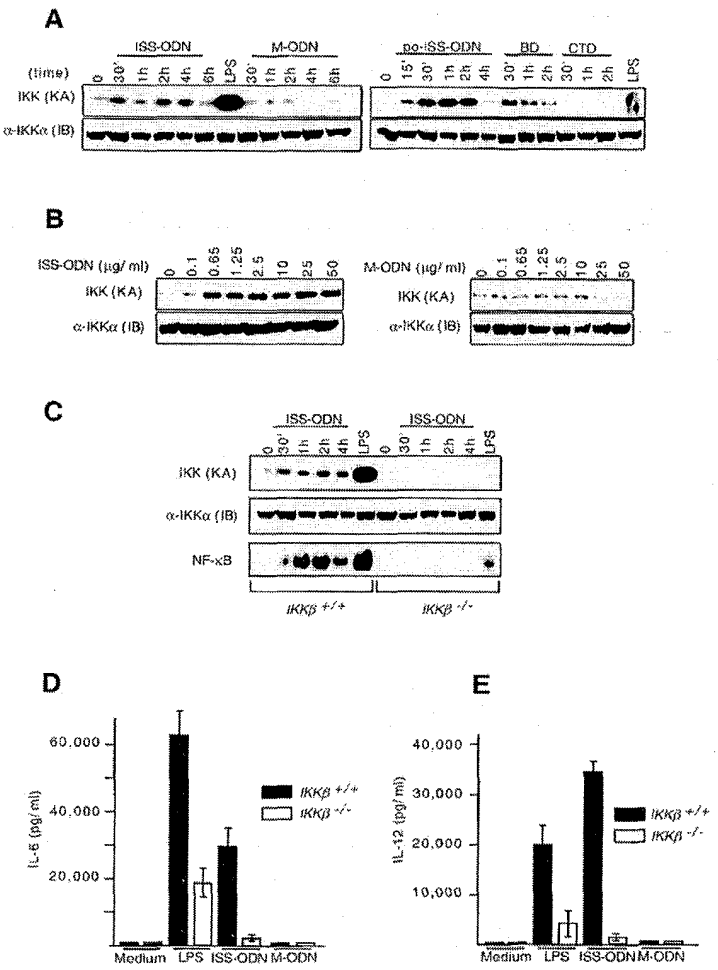

Figure 3. Involvement of IKK in NF-κB Activation by ISS-ODN and Bacterial DNA

(A) Left panel: BMDM from wt mice were treated with ISS-ODN (5 μg/ml) M-ODN (5 μg/ml) for time periods as indicated. Right panel: BMDM from wt mice were treated with po-ISS-ODN (5 μg/ml), bacterial DNA (BD, 5 μg/ml), or calf thymus DNA (CTD, 5 μg/ml) for the time periods as indicated. The cell lysates were assayed for IKK activity (KA) by an immune complex kinase assay. The recovery of IKK was determined by immunoblotting (IB) with anti-IKKα antibodies.

(B) BMDM from wt mice were treated for 2 hr with ISS-ODN (left panel) or M-ODN (right panel) at different concentrations. The cell lysates were assayed for IKK kinase activity (KA) by an immune complex kinase assay. The recovery of IKK was determined as mentioned above.

(C) BMDM from wt and *IKKβ*$^{-/-}$ *Tnfr1*$^{-/-}$ mice were treated with ISS-ODN (5 μg/ml). At the indicated time points, cells were lysed, and IKK immune complex was isolated by anti-IKKα antibodies to measure IκB kinase activity (KA). Recovery of IKK was determined by immunoblotting (IB). Cells were stimulated with LPS (10 μg/ml) for 30 min. NF-κB DNA binding activity was measured by an electrophoretic mobility shift assay (EMSA).

(D and E) IKKβ is required for induction of IL-6 and IL-12 in response to ISS-ODN. BMDM isolated from wt or *IKKβ*$^{-/-}$ *Tnfr1*$^{-/-}$ mice were treated with ISS-ODN (5 μg/ml), LPS (1 μg/ml), or left untreated for 24 hr. IL-6 and IL-12 levels were determined by ELISA.

upon incubation of IKKα with DNA-PK in the presence of ISS-ODN, but not beyond the level found with DNA-PK alone (Figure 6A, lane 6 vs. lane 5). To further confirm the activation of IKKβ by DNA-PK, we performed a coupled-kinase assay. Recombinant IKKβ was preincubated with DNA-PK in the presence or absence of ISS-ODN followed by immunoprecipitation of IKKβ and IκB kinase activity was measured. Consistent with the results described above, DNA-PK only activates IKKβ in the presence of ISS-ODN (data not shown). We next determined whether DNA-PK phosphorylates IKKβ. Recombinant catalyticly inactive IKKβ [IKKβ (KA)] purified from Sf9 cells was incubated with or without DNA-PK, in the presence or absence of ISS-ODN. As shown in Figure 6B, DNA-PK phosphorylated IKKβ (KA) when incubated with ISS-ODN.

Discussion

Infection of the mammalian host with bacterial, fungal, and viral pathogens results in rapid activation of innate immunity, which provides a rapid defense and at the same time alerts the adaptive immune system to the presence of pathogens (Medzhitov and Janeway, 1997). The innate immune response to invading pathogens involves the effective and rapid recognition of highly conserved and usually repetitive microbial structural patterns such as those found in polysaccharides, lectins, LPS, and dsRNA (Medzhitov and Janeway, 1997). This recognition usually occurs through pattern recognition receptors such as those that belong to the Toll family (Poltorak et al., 1998; Aderem and Ulevitch, 2000). It has been speculated that bacterial DNA and its synthetic analogs (i.e., ISS-ODNs), due to their relatively high content of nonmethylated CpG dinucleotides are similarly recognized as foreign structures by the innate immune system of the invaded host (Klinman et al., 1999; Wagner, 1999). The recognition mechanism, however, remained enigmatic.

The development of innate immunity appeared relatively early in evolution preceding the appearance of adaptive immunity, a protective mechanism unique to vertebrates (Hoffmann et al., 1999). Analysis of innate immune responses to bacterial and fungal pathogens in

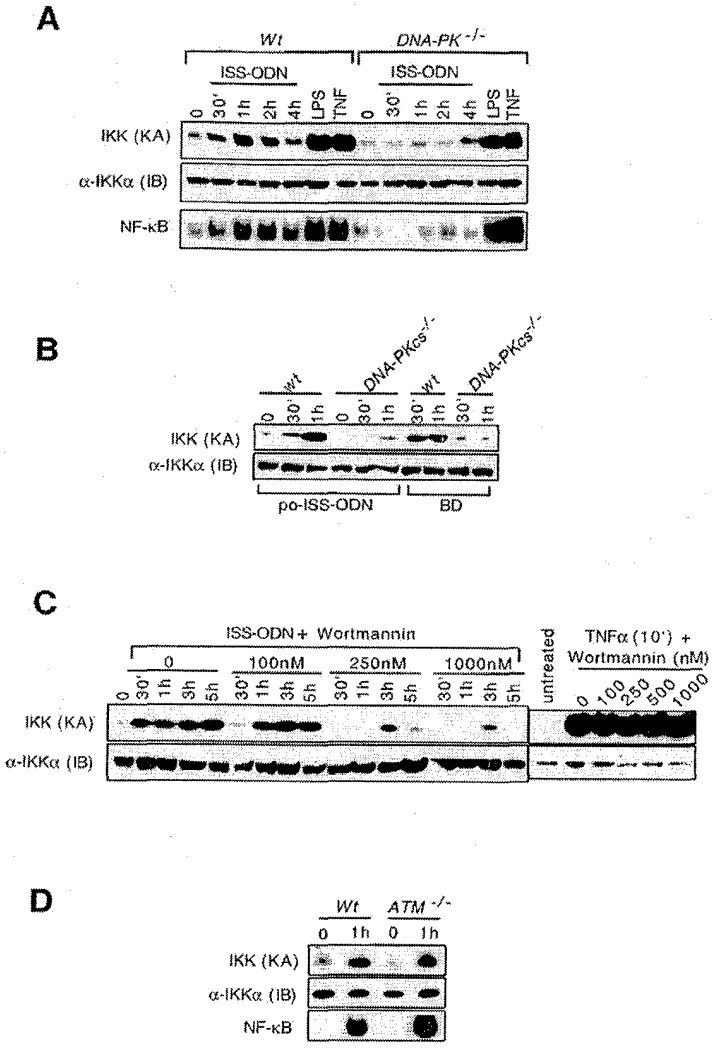

Figure 4. DNA-PKcs Activates IKK by ISS-ODN and Bacterial DNA

(A) BMDM from wt or *DNA-PKcs*$^{-/-}$ mice were treated with ISS-ODN (5 μg/ml) for various time periods, LPS (10 μg/ml for 30 min) or TNFα (10 ng/ml for 10 min). At the indicated time points, cells were lysed and IKK activity was measured by immune complex kinase assays (KA). Recovery of IKK was determined by immunoblotting (IB). NF-κB DNA binding activity was determined by EMSA.

(B) BMDM from wt or *DNA-PKcs*$^{-/-}$ mice were treated with po-ISS-ODN (5 μg/ml) or bacterial DNA (BD, 5 μg/ml). At the indicated time points, cells were lysed, IKK activity and IKK recovery were analyzed as described above.

(C) BMDM from wt mice were treated with ISS-ODN (5 μg/ml) or TNFα (10 ng/ml) as a control, in the presence of different concentrations of Wortmaninn (Wm) for the indicated time periods, after which IKK activity was measured by immune complex kinase assay (KA). Recovery of IKK was determined by immunoblotting (IB).

(D) BMDM isolated from wt or *ATM*$^{-/-}$ mice were treated with ISS-ODN for 1 hr. Cells were lysed and IKK activity, IKK recovery, and NF-κB DNA binding activity were analyzed as described above.

Drosophila has implicated the involvement of signaling pathways that are highly similar to those that activate AP-1 and NF-κB transcription factors in vertebrates (Hoffmann et al., 1999). The role of AP-1 and NF-κB in the control of the innate immune response in mammals is well recognized (Baldwin, 1996; May and Ghosh, 1998; Chu et al., 1999). It is also well established that IKK is activated by dsRNA, viral infection, or LPS, and is required for cytokine or chemokine production (Chu et al., 1999; Karin and Delhase, 2000).

The results presented here shed new light on the mechanism by which ISS-ODNs activate innate immunity by demonstrating that: (1) DNA-PKcs is required for induction of IL-6 and IL-12, (2) DNA-PKcs activates IKKβ, which is also required for IL-6 and IL-12 induction by ISS-ODN, and (3) DNA-PKcs and IKK activities are more efficiently stimulated by biologically active ISS-ODNs than by other ODNs that lack immunostimulatory activity.

Several lines of evidence indicate that DNA-PK is involved in the cellular response to DNA ds breaks (Smith and Jackson, 1999). The protein kinase activity of DNA-PK is stimulated by DNA ends (Carter et al., 1990; Gottlieb and Jackson, 1993; Leuther et al., 1999; Hammarsten et al., 2000). In addition, DNA with blunt ends, 5′ overhanging ends, or 3′ overhanging ends all activate DNA-PK with similar efficiency (Gottlieb and Jackson, 1993), whereas ds DNA with hairpin ends and supercoiled DNA are inactive (Smider et al., 1998; Hammarsten et al., 2000). Other studies revealed that DNA-PKcs binds both ds and ss DNA (Leuther et al., 1999; Smith and Jackson, 1999; Hammarsten et al., 2000). The minimal length of ds-DNA required for efficient DNA-PKcs binding is no less than 12 bp while the optimal length of ss DNA needed for efficient DNA-PKcs activation is between 5 and 10 nucleotides (Leuther et al., 1999; Hammarsten et al., 2000).

Our results here have identified a novel function for

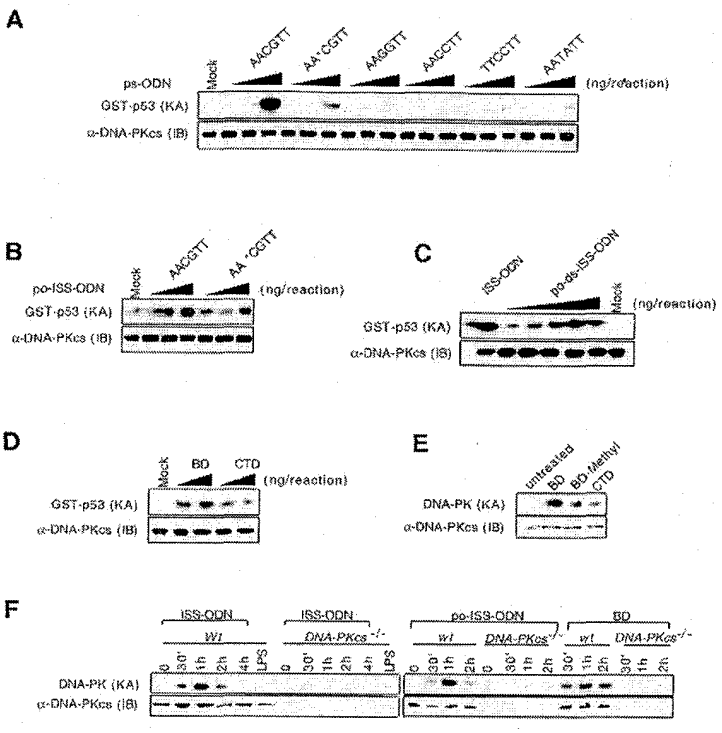

Figure 5. ISS-ODN and Bacterial DNA Activate DNA-PK In Vitro and In Vivo

(A) The ODNs; ISS-ODN (...AACGTT), methylated ISS-ODN at the ^{5}C position of the CpG dinucleotode (...AA*CGTT) or mutated ODN (M-ODNs: ...AAGGTT..., ...AACCTT... or AA TATT) at a concentration of 0, 0.1, 0.3, or 1 ng/reaction were incubated with affinity-purified DNA-PK (Promega), 0.5 μg of GST-p53 (1–70), and 3.3 μCi of γ-^{32}P-ATP at 30°C for 30 min. The reactions were stopped by addition of 4× sample buffer and the samples were boiled, separated on 9% SDS-PAGE, transferred on a PVDF membrane, and visualized by autoradiography at −80°C for 1 hr.

(B) The po-ODNs ISS-ODN (...AACGTT) and methylated po-ISS-ODN (...AA*CGTT) at a concentration of 20, 50, or 100 ng/reaction were incubated with affinity-purified DNA-PK. DNA-PK activity was determined as described above.

(C) The ISS-ODN (AACGTT) at concentrations of 0.3 and 1 ng/reaction was compared to stimulate DNA-PK activity to the po-ds-ISS-ODN (AACGTT) at concentrations of 0.2, 1, 2, 5, 10 ng/reaction. DNA-PK activity was determined as described above.

(D) Equimolar amounts of bacterial DNA (BD) or calf thymus DNA (CTD) as measured by γ-^{32}P labeling of free 5′ ends (4.1×10^6 and 4.2×10^6 cpm per 1 μg of DNA, respectively). 5 ng of each DNA, roughly equal to 2×10^4 cpm, was incubated with affinity-purified DNA-PK and 3.3 μCi of γ-^{32}P-ATP at 30°C for 30 min. The DNA-PK activity was determined as described above.

(E) BMDM isolated from wt mice were treated with bacterial DNA (BD, 2.5 μg/ml), methylated bacterial DNA (2.5 μg/ml), or calf thymus DNA (CTD, 2.5 μg/ml) for 1 hr and then lysed. DNA-PK activity was determined as described above.

(F) BMDM isolated from wt and *DNA-PKcs*$^{-/-}$ mice were treated with ISS-ODN (ps-ss) (5 μg/ml), po-ISS-ODN (ss) (5 μg/ml), bacterial DNA (BD, 2.5 μg/ml), LPS (10 μg/ml), or left untreated for the indicated time periods. Cells were then lysed and the 100 μg of lysates were assayed for DNA-PK activity by an immune complex kinase assay (KA) using anti-DNA-PKcs monoclonal antibodies (mAb) and GST-p53 (1–70) as a substrate. Recovery of DNA-PKcs was determined by immunoblotting (IB).

DNA-PK, i.e., interaction and activation by fragments of bacterial DNA and ISS-ODN (Figure 5). Optimal activation of DNA-PKcs by ISS-ODNs appears to be sequence specific since mutations within the CpG motif which impair immunostimulatory activity (Krieg et al., 1995) also reduce the ability to activate DNA-PK (Figure 5). Nevertheless, even biologically inactive ODNs retain the ability to activate DNA-PK, an effect mediated by DNA ends. Using BMDM from DNA-PKcs-deficient mice or high concentrations of Wm sufficient for DNA-PK inhibition, we obtained genetic and biochemical evidence that the activation of DNA-PK by ps and po ISS-ODNs as well as bacterial DNA is required for induction of IL-6 and IL-12 (Figure 1). Although DNA-PK was also activated by methylated ISS-ODN and methylated bacterial DNA (Figure 5), which have reduced biological activity, this activation was weaker than that observed for nonmethylated ISS-ODN or bacterial DNA (Figure 5). It is likely, however, that DNA-PKcs, although essential for activation of innate immunity by ISS-ODN is not sufficient for complete discrimination between methylated vs. nonmethylated and mammalian vs. bacterial sequences. Additional components may exist that further increase the discriminatory ability of this molecular recognition system. The MyD88 is a potential component of this auxiliary recognition system as it was recently shown to mediate signaling by ISS-ODN and to be required for ISS-induced IKK activation (Hacker et al., 2000). However, the molecular link between DNA-PK and MyD88 has yet to be established.

Our results also demonstrate that in addition to its requirements for cytokine induction, DNA-PKcs is required for NF-κB activation in response to ISS-ODN. Although DNA-PK can phosphorylate sites at the C terminus of IκBα, it is not directly involved in induction of IκBα degradation (Basu et al., 1998; Liu et al., 1998). Rather, DNA-PKcs is likely to affect NF-κB activation via IKK. The defect in NF-κB activation in DNA-PKcs-deficient cells correlates with their inability to rapidly activate the IKK complex in response to ISS-ODN. The activation of IKK by ISS-ODN is largely dependent on IKKβ phosphorylation, as was shown for other pro-inflammatory stimuli (Chu et al., 1999; Li et al., 1999; Tanaka et al., 1999). Although DNA-PKcs was first identified as a nuclear protein (Smith and Jackson, 1999), several recent reports demonstrated that DNA-PKcs is also located in the cytoplasm (Carter et al., 1990; Danska et al., 1996; Nilsson et al., 1999; Koiker et al., 1999). Thus, it is plausible that ISS-ODN activates the catalytic subunit of DNA-PK in the cytoplasm, which in turn phos-

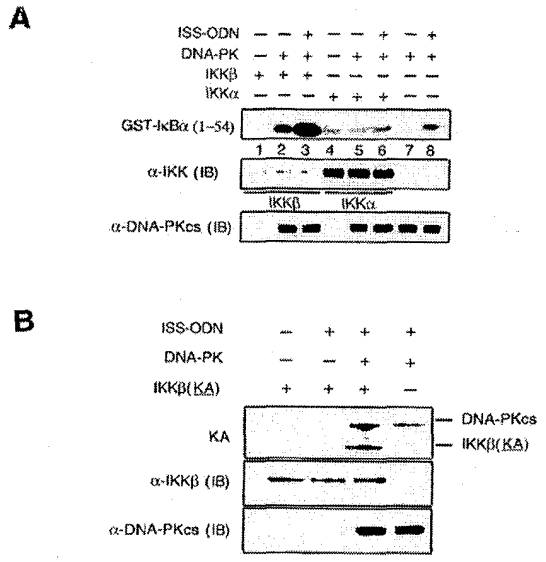

Figure 6. DNA-PK Activates IKKβ through Phosphorylation

(A) DNA-PK activates IKKβ but not IKKα. IKKβ (1 μl, 1:40 dilution) or IKKα (1 μl) purified from Sf9 cells (Zandi et al., 1998) was incubated with DNA-PK (1 μl, 1:3 dilution), 0.5 μg of GST-IκBα (1–54) and 3 μCi of γ-^{32}P-ATP in the presence or absence of ISS-ODN (2 ng) at 30°C for 30 min. The reaction was stopped by addition of 4× sample buffer. The samples were boiled, loaded on a 9% SDS-PAGE, transferred onto a PVDF membrane, and visualized by autoradiography. The presence of IKKα, IKKβ, or DNA-PK in various reactions was determined by immunoblotting (IB).

(B) DNA-PK phosphorylates catalytic inactive subunit of IKKβ [IKK (KA)]. IKKβ (KA) (3 μl) purified from Sf9 cells (Zandi et al., 1998) and 10 μCi of γ-^{32}P-ATP were incubated with DNA-PK (1 μl), or left alone in the presence or absence of ISS-ODN (2 ng) at 30°C for 45 min. The reaction was stopped by addition of 4× sample buffer. The samples were loaded, transferred onto a PVDF membrane, and visualized by autoradiography. The presence of IKKβ (KA) or DNA-PK in the reactions was determined by immunoblotting (IB).

phorylates and activates the IKKβ subunit of IKK leading to NF-kB activation. Although the absence of DNA-PKcs results in a dramatic decrease in IKK activation by ISS-ODN or bacterial DNA, some residual activity can be observed (Figure 4). This residual activity can be derived from ISS-induced TNFα production, which is not fully dependent on NF-κB, or can be due to the operation of the previously mentioned auxiliary recognition system.

DNA-PK is a member of the DNA repair machinery (so-called "caretaker") and has an essential role in the maintenance of genome stability (Smith and Jackson, 1999). DNA-PK plays a pivotal role in DNA ds breaks repair and therefore in programmed antigen receptor rearrangements during B and T cells development (Carrol and Bosma, 1991; Gottlieb and Jackson, 1993; Kirchgessner et al., 1993; Hartley et al., 1995; Danska et al., 1996; Kurimasa et al., 1999; Smith and Jackson, 1999). DNA-PKcs null mice are hypersensitive to ionizing radiation and develop severe combined immune deficiency (Kurimasa et al., 1999; Smith and Jackson, 1999). Thus, the requirement of DNA-PK in adaptive immunity is well established. Our results indicate that DNA-PKcs is also required for activation of innate immunity by ISS-ODN. This finding fits nicely with previous reports in which DNA-PKcs has been found to be degraded, apparently via a proteosomal mechanism, during herpes simplex virus (HSV) type I infection of mammalian cells (Lees-Miller et al., 1996; Parkinson et al., 1999). As a result, HSV was shown, in these studies, to replicate more efficiently in a cell line with undetectable DNA-PKcs than in a wt control cell line (Lees-Miller et al., 1996; Parkinson et al., 1999). Therefore, DNA-PKcs may also be involved in mounting innate anti-viral responses. Our results explain how a deficiency in DNA-PKcs results in defective activation of innate immunity.

In conclusion, DNA-PK plays a pivotal role in two different responses to DNA: (1) activation of DNA repair and recombination systems, and (2) activation of innate immunity. DNA-PK is involved in protecting the host genome from danger imposed by intrinsic DNA damage such as DNA ds breaks generated by ionizing radiation or produced during immunoglobulin gene rearrangement. In addition, DNA-PK is involved in protecting the host from a danger imposed by invading bacterial DNA (e.g., intracellular infection). Thus, DNA-PK provides a link between genome defense (DNA repair machinery) and host defense (innate immunity).

Experimental Procedures

Animals, Cell Culture, DNAs, ODNs, and ELISA

DNA-PKcs$^{-/-}$ and their wt control mice on the 129 genetic background were generated by Dr. G. C. Li and bred at Memorial Sloan-Kettering Cancer Center, New York, NY. *IKK*β $^{-/-}$ *Tnfr1*$^{-/-}$ mice were generated by Drs. Z.-W. Li and M. Karin (unpublished data). *ATM*$^{-/-}$ mice on the C57BL/6 background were generated and bred by Dr. Y. Xu (UCSD) as was previously described (Xu et al., 1996) while their wt controls were purchased from Jackson Laboratories (Bar Harbor, ME). BMDMs from wt, *DNA-PKcs*$^{-/-}$ mice, *IKK*β $^{-/-}$ *Tnfr1*$^{-/-}$, and *ATM*$^{-/-}$ mice were prepared as was previously published (Martin-Orozco et al., 1999), maintained in DMEM with 10% FBS, antibiotics, and 20% L-cell medium, and cultured for 7–10 days to allow them to mature. Prior to use, BMDM were seeded (2.5 × 10^5/well in triplicate) in 96 well plates and then treated with LPS (1 μg/ml), ISS-ODN (5 μg/ml) or M-ODN (5 μg/ml), po-ISS-ODN (10 μg/ml) or po-ds-ISS-ODN (10 μg/ml), LPS-free, ultra pure bacterial DNA (*E. coli*, Sigma) (15 μg/ml) or methylated bacterial DNA or LPS-free, ultra pure calf thymus DNA (Sigma) (15 μg/ml). Methylation of bacterial DNA was performed by SssI methylase (Biolab, Boston, MA) (15 μg/ml) following manufacturer's instruction. Where indicated, the PI3K inhibitor wortmannin (Wm), at various concentrations, was added to ISS-ODN or LPS stimulated BMDMs. After 24 hr in culture, the supernatants were collected and assayed for IL-6 and IL-12 levels by ELISA kits (PharMingen, San Diego, CA).

Most of the experiments described in this study were performed with LPS free, single-stranded (ss), 22 mer long, phosphothioate (ps) ODNs. In some experiments, ss and double-stranded (ds) 22 mer long phosphodiester (po) ODNs were used. (Trilink, San Diego, CA). The sequences of the ODNs used in this study are as follow:

- ISS-ODN (1), 5′-TGACTGTGAACGTTCGAGATGA-3′
- ISS-ODN (2), 5′-TGACTGTGAACGTTAGAGATGA-3′
- Methylated ($^{5\text{-methyl}}$C) ISS-ODN, 5′-TGACTGTGAA*CGTTAGAGATGA-3′
- Mutated (M)-ODN, 5′-TGACTGTGAAGGTTAGAGATGA-3′
- Control-ODN (1), 5′-TGACTGTGAACCTTAGAGATGA-3′
- Control-ODN (2), 5′-TGACTGTGTTCCTTAGAGATGA-3′
- Control-ODN (3), 5′-TGACTGTGAATATTAGAGATGA-3′

Kinase Assays and Immunoblotting

Kinase assays and immunoblotting were performed according to Li et al. (1999). Briefly, BMDM were treated with ISS-ODN (5 μg/ml),

M-ODN (5 μg/ml) on ps and po backbones as indicated, LPS-free bacterial DNA or methylated bacterial DNA (5 μg/ml), LPS-free calf thymus DNA (5 μg/ml), LPS (10 μg/ml), or TNFα (10 ng/ml) for the indicated time periods. Cell lysates were prepared and normalized by immunoblotting (IB) with anti-IKKα polyclonal antibodies (Santa Cruz, Santa Cruz Biotech Inc., CA), anti-IKKβ polyclonal antbodies (Santa Cruz), or anti-DNA-PKcs monoclonal antibodies (NeoMarker, CA). IκB kinase (IKK) complex or DNA-PK complex from 100 μg of the lysates were immunoprecipitated by anti-IKKα or by anti-DNA-PKcs antibodies. The kinase activities (KA) were determined by a kinase assay using the N terminus of IκBα (for IKK) or the N terminus of p53 (for DNA-PK) as a substrate as previously described (Wang and Eckhart, 1992; Li et al., 1999; Hammarsten et al., 2000).

The in vitro DNA-PK phosphorylation assay was performed according to Hammarsten et al. (2000) with modification. Briefly, affinity-purified DNA-PK (Promega, MO) was incubated with various DNA preparations (described below), 0.5 μg of GST-p53 (1–70) and 3.3 μCi of γ-^{32}P-ATP in a 20 μl reaction buffer (10 mM Tris-Cl, 5 mM $MgCl_2$, 0.3 mM EDTA, and 10 μM ATP) at 30°C for 30 min. The reaction was stopped by addition of 4× loading buffer. The samples were boiled, loaded on 9% SDS-PAGE, transferred onto a PVDF membrane and visualized by autoradiography.

The ODNs (ps-ss) used include an ISS-ODN with an active CpG motif (AACGTT), a methylated ISS-ODN at the ^{5}C of the CpG dinucleotide (AA*CGTT), and various control ODNs. These ODNs were incubated at concentrations of 0, 0.1, 0.3, or 1 ng/reaction. The po-ISS-ODN was incubated at a concentration of 20, 50, or 100 ng/reaction. The po-ds-ISS-ODN was incubated at a concentration of 0, 0.5, 1, 2, 5, or 10 ng/reaction. The bacterial or calf thymus DNAs were each incubated at a concentration of 1, 2, or 5 ng/reaction. Electrophoretic gel mobility shift assay (EMSA) was performed as previously described (Chu et al., 1999; Li et al., 1999). In vitro IKK kinase assays were performed using purified IKK derived from Sf9 insect cell lysates as was previously described (Zandi et al., 1998).

RT-PCR and Northern Blots

Total cellular RNA was isolated from spleen or liver of wt or DNA-PKcs$^{-/-}$ mice injected with ISS-ODN (50 μg), po-ISS-ODN (100 μg), po-ds-ISS-ODN (100 μg), bacterial DNA (100 μg), or calf thymus DNA (100 μg), using a RNA isolation kit (Stratagene, San Diego, CA), and subjected to RT-PCR. First strand cDNA preparation and PCR amplification were performed using the SuperScript preamplification system (Gibco BRL, Gaithersburg, MD) and AdvanTaq Plus DNA polymerase (Clontech, San Francisco, CA), respectively. The primer sequences used were as follows:

- IL-6: (sense) 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′; (antisense) 5′-CACTAGGTTTGCCGAGTAGATCTC-3′
- IL-12p40: (sense) 5′-GGGACATCATCAAACCAGACC-3′; (antisense) 5′-GCCAACCAAGCAGAAGACAGC-3′
- GAPDH: (sense) 5′-ACCACAGTCCATGCCATCAC-3′; (antisense) 5′-TCCACCACCCTGTTGCTGTA-3′

PCR reactions were performed under the following conditions by appropriate cycling number (94°C: 30 s; 65°C: 30 s; 68°C: 30 s). PCR products were visualized by electrophoresis on 1.5% TAE agarose gels after being stained with ethidium bromide. BMDM isolated from either wild-type or DNA-PKcs-deficient mice were treated with ISS-ODN (10 μg/ml), LPS (10 μg/ml), or left untreated for 6.5 hr. Total RNA was isolated and 10 μg of total RNA was separated on 1% agarose gel and then transferred onto a nylon membrane. The membrane was probed with α-^{32}P-dCTP-labeled IL-6 or IL-12 or GAPDH cDNA (generated by RT-PCR as described above) followed by autoradiography.

Methylation and Labeling of Free Ends of Genomic DNAs

When indicated, 100 μg of ultra pure bacterial DNA (*E. coli*, Sigma) was incubated with or without 200 units SssI methylase (Biolab) in a 200 μl reaction buffer according to manufacturer's instruction at 37°C for 3 hr and then extracted with phenol/chloroform. The bacterial DNA was precipitated with ethanol and dissolved in TE buffer. 1 μg of mock and methylated bacterial DNA was further incubated with 10 units of BstU1 at 60°C for 4 hr, loaded on 1% agarose gel, and visualized by EB staining for the absence or presence of digest products in the methylated and in the nonmethylated bacterial DNA, respectively (data not shown).

For the DNA-PK assay, we measure the free ends in *E. coli* and calf thymus DNAs by labeling the 5′ free ends of both the DNA preparations. Thus, 0.2 μg of either bacterial or calf thymus DNAs were incubated with 15 units of T4 PNK (Stratagene, San Diego, CA) and 100 μCi of γ-^{32}P-ATP in a 20 μl of reaction at 37°C for 3 hr. To purify the labeled DNAs from the γ-^{32}P-ATP excess, the samples were loaded onto SephdexG50 column (Stratagene San Diego, CA) after the reaction was stopped. 1 μl of labeled DNA was used to measure radioactivity which yielded $4.1 \times 10^{6} \pm 2 \times 10^{4}$ cpm/1 μg for bacterial DNA and $4.2 \times 10^{6} \pm 5.2 \times 10^{4}$cp m/1 μg for calf thymus DNA.

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